Development of optimal guideline for effective dendritic cell immunotherapy in mouse melanoma

Han Kyoung Cho

Department of Medicine

The Graduate School, Yonsei University

Development of optimal guideline for effective dendritic cell immunotherapy in mouse melanoma

Directed by Professor Min-Geol Lee

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Han Kyoung Cho

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This certifies that Doctoral Dissertation of Han Kyoung Cho is approved.

Thesis Supervisor : Min-Geol LEE

Thesis Committee : Kwang Hoon Lee

Thesis Committee : In Hong Choi

Thesis Committee : Si Young Song

Thesis Committee : Jong Baeck Lim

The Graduate School

Yonsei University

June 2007

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

DCs: dendritic cells

LNs: lymph nodes

NK: natural killer

CM: complete medium

Ab: antibody

RT: room temperature

Ag: antigen

mAb: monoclonal antibody

APC: antigen-presenting cell

CTL: cytotoxic T lymphocyte

LPS: lipopolysaccharide

TLR: Toll-like receptor

ABSTRACT

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Mouse bone marrow-derived dendritic cells (DCs) were stimulated with tumor antigen alone or tumor antigen plus cocktail (anti-CD40 antibody+TNF-α+IL-1β) for 8 hours, 24 hours or 48 hours, and the characteristics of DCs, such as surface molecules (CD40, CD80, CD86, MHC class II, and CCR7) on DCs, cytokines (IL-12, IFN-γ, and IL-10), DCs-induced T cell proliferation *in vitro*, and the production of IFN-γ by those T cells, were evaluated.

Mice with melanoma were then treated with DCs stimulated with tumor antigen alone or tumor antigen plus cocktail for 8 hours or 48 hours. The tumor size and the survival rate of mice were then evaluated. In order to develop optimal guideline toward the determination of proper cell type for DCs immunotherapy in mouse melanoma, the functional characteristics of DCs were matched with the clinical efficacy of DCs immunotherapy.

1) Beneficial clinical effects such as a reduction of tumor size and an increased survival rate were best observed in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail.

2) The single prominent characteristic of DCs stimulated for eight hours with tumor antigen plus cocktail was an elevated IL-12 secretion. The cytokine IL-12 was not secreted by other DCs.

Consequently, proper production of IL-12 was found to be an important functional guideline for DCs immunotherapy in mouse melanoma.

Key words : dendritic cell, immunotherapy, melanoma, optimal guideline

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I. INTRODUCTION

Malignant melanoma is a potentially lethal cancer that arises from melanocytes present in the skin, mucosa, or the epithelial surfaces of the eyes and ears, and its incidence has increased substantially over the past two decades[1, 2]. Although primary tumor excision can sometimes achieve complete remission, most melanomas are beyond surgical margins when diagnosed, and are usually resistant to chemotherapy and radiotherapy[3]. Consequently, many other therapeutic modalities are being investigated, and one of them being dendritic cell-based immunotherapy[4, 5].

Dendritic cells (DCs) are one of the most potent antigen-presenting cells[6]. DCs residing in the skin or mucosa are in their immature state and have great phagocytic capacity. After antigen uptake, DCs undergo maturation, noted by a downregulated antigen uptake and increased antigen processing ability. They then migrate to the regional lymph nodes (LNs) where they present antigen to naive T cells, which generate a potent host immunity to the antigen[7]. It has been reported that efficient antitumor immunity is generated by immature DCs because immature DCs phagocytose both necrotic and apoptotic tumor cells, and can mature *in vivo*[8]. The abilities of DCs to phagocytose tumor cells *in vivo* and migrate to regional LNs 24 hours after injection have also been demonstrated. These findings suggest that DCs can be used efficiently in cancer immunotherapy. Such DC-based immunotherapies have been attempted in mice and humans, and positive results have been obtained with many tumors, especially with malignant melanoma, B cell lymphoma, colorectal cancer, and prostate cancer[9-12].

Despite some favorable results, however, DCs immunotherapy has yet to show acceptable, reasonable clinical effects. To enhance clinical effects, DCs immunotherapy must have the ability to activate some effector cells such as cytotoxic T cells, helper T cells, B cells, natural killer (NK) cells, NK T cells or γ:δ T cells. In order to succeed in DCs immunotherapy, sufficient considerations of both dendritic cellular factors and non-dendritic cellular factors are essential[13]. Nestle et al. emphasized three parameters need to be

taken into account using DCs immunotherapy; the choice of antigen and adjuvant, the choice of injection site (localization) and the choice of timing and dosing[14]. There are many issues of DCs immunotherapeutic challenges, including; source and *ex vivo* manipulation of DCs; antigen preparation and loading; and, route of administration. In addition, methods of measuring the immune and clinical response need to be standardized and adopted using state of the art procedures[14]. Currently, there is no consensus among the literature regarding the optimal guideline to follow in the development of DCs immunotherapy. A successful clinical response with DCs immunotherapy may not entirely depend on simply finding of optimal guideline of DCs. However, establishing optimal guideline of DCs for immunotherapy must be the first step to successful DCs immunotherapy.

In this study therefore, mouse bone marrow-derived DCs were prepared and were further stimulated with maturation factors for 8, 24 and 48 hours. Then these several different types of DCs were injected into mice with malignant melanoma as a form of immunotherapy. By means of matching the functional characteristics of DCs with the clinical results obtained by DCs immunotherapy, we attempted to define optimal guideline of DCs for DCs immunotherapy in mouse melanoma.

II. MATERIALS AND METHODS

1. Mice

C57BL/6 female mice (6-10 weeks old) were purchased from Daehan Biolink (Seoul, Korea) and housed in pathogen-free units at the Yonsei Medical Research Center.

2. Media and Cytokines

Complete medium (CM) consisting of RPMI 1640 (Gibco, BRL, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Gibco), 100IU/㎖ penicillin (Gibco), 100 ㎍/㎖ streptomycin (Gibco), 0.1mM nonessential amino acids (Sigma, Saint Louis, MO), 1mM sodium pyruvate (Sigma), 10mM HEPES (Sigma), and 50μM 2-mercaptoethanol (Sigma). DMEM (Gibco) and RPMI 1640 were used to culture hybridoma cell lines. Recombinant mouse GM-CSF (rmGM-CSF) or recombinant mouse IL-4 (rmIL-4) were purchased from Endogen (Woburn, MA) and used to generate DCs. Maturation cocktail containing anti CD-40 antibody, TNF-α and IL-1β was used.

3. Preparations of Bone Marrow-derived DCs

Bone marrow cells were obtained from the tibias and femurs of C57BL/6 mice (6-8 weeks old) and depleted of erythrocytes using commercial lysis buffer (Sigma). Bone marrow cells were then treated with a mixture of

antibodies (Abs) [anti-CD4 (GK1.5, TIB-207), anti-CD8 (53-6.72, TIB-105), anti-B220 (RA3-3A1/6.1, TIB-146), anti-I-$A^{b,d,q}$ & I-$E^{d,k}$ (M5/114.15.2, TIB-120), anti-erythrocytes, neutrophils & B cells (J11d.2, TIB-183); all were obtained from the ATCC] for 30min at 4℃, and then treated with rabbit complement (Cedarlane, Ontario, Canada) for 1hour at 37℃. Cells were then layered onto lympholyte M (density: 1.0875±0.0010g/㎖, Cedarlane) gradients, centrifuged, and low-density interfaces were collected. Cells were washed three times with CM, and then incubated in CM supplemented with 10ng/㎖ GM-CSF and 10ng/㎖ IL-4 in 24 well plates at 7-10×10^5/well. On the second day of culture, floating cells were gently removed and fresh medium containing GM-CSF and IL-4 was replaced. On day four, floating cells were harvested and fresh medium containing GM-CSF and IL-4 was replaced. These harvested cells were then plated in new 24 well plates in CM supplemented with 10ng/㎖ GM-CSF and 10ng/㎖ IL-4 at 5×10^5/㎖. On day six, non-adherent and loosely adherent proliferating DCs were collected and counted.

4. Flow Cytometric Analysis

To identify surface molecules expressed on DCs, cells were harvested on day 5 (D5), 6 (D6) and 7 (D7). Cells (6-10×10^5) were washed twice with 0.4% BSA/PBS and stained for 30min at 4℃ with monoclonal Abs against CD11c (Pharmingen, San Diego, CA), CD80 (Pharmingen), CD86 (Pharmingen) and MHC class II (I-$A^{b,d,q}$ & I-$E^{d,k}$). Hamster IgG (Pharmingen)

and rat IgG2a (Pharmingen) were used as isotype control. After two washes with 0.4% BSA/PBS, secondary staining was performed using FITC-conjugated $F(ab')_2$ goat anti-rat Ig's (Biosource, Camarillo, CA) and FITC-conjugated anti-hamster IgG (Pharmingen). After 30min, cells were washed twice with 0.4% BSA/PBS and resuspended in 400 ㎕ of 0.4% BSA/PBS. Propidium iodide (Sigma) was added to exclude dead cells from the analysis. Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA).

5. Experimental Design

In vitro

Prepared DCs were grouped by an additional stimulation method and time. Group 1, group 2, and group 3 were incubated for 8 hours, 24 hours and 48 hours, respectively with no additional stimulation. Group 4, 5, and 6 were incubated for 8 hours, 24 hours and 48 hours, respectively, under the stimulation of tumor antigen. Finally, group 7, 8, and 9 were incubated for 8 hours, 24 hours and 48 hours, respectively, with stimulation of tumor antigen + cocktail (anti CD-40 antibody, TNF-α and IL-1β).

In vivo

A malignant melanoma model was made by inoculating 2.5×10^5 B16F10 cells in 100 ㎕ PBS subcutaneously into C57BL/6 mice (6-10 weeks old). Six days later 50 mice were divided into five groups: 1) injected with PBS control

(10 mice, Group Ⅰ), 2) injected with DCs stimulated for eight hours with tumor antigen (10 mice, Group Ⅱ), 3) injected with DCs stimulated for 48 hours with tumor antigen (10 mice, Group Ⅲ), 4) injected with DCs stimulated for eight hours with tumor antigen plus cocktail (10 mice, Group Ⅳ), 5) injected with DCs stimulated for 48 hours with tumor antigen plus cocktail (10 mice, Group Ⅴ).

6. Measurement of Tumor Size and Survival Rate

After DCs immunotherapy of each grouped mice, tumor long and short axes were measured with calipers (Mitutoyo, Japan) at two to three day intervals. Tumor volumes were calculated using the formula: $V=1/2\times A^2\times B$, where A is the length of the short axis and B that of the long axis. Surviving mice were counted until all group I mice succumbed. Survival rates were recorded as percentage survivals.

7. Delayed Type Hypersensitivity Assay (DTH)

Seven days after the third DCs injection, each mouse received 300 ㎍ of tumor lysates (suspended in 50 ㎕ of PBS) subcutaneously injected into the right footpad area. As a negative control, an identical amount of PBS was also injected into the left footpad of the same mouse. After 48 hours, the extent of swelling was measured using calipers (Mitutoyo). Results were reported as the differences in thickness (in millimeters) between both footpads.

8. T cell Proliferation Assay

Splenocytes were obtained from each experimental group and commercial erythrocyte lysis buffer was added. After five min, the reaction was stopped by adding CM, then washed twice with CM. Triplicate samples of 1.5×10^5 cells in CM were seeded in 96 well round-bottom plates (Corning, NY). Splenocytes (responder; R) were stimulated with B16F10 cells (stimulator; S) treated with mitomycin c and irradiation (30,000 rad) at a S:R ratio of 1:50. Splenocytes from untreated mice were used as negative control. The cells were incubated at 37℃ for 96 hours, then 1μCi/well [^{3}H] thymidine was added for another 16 hours. Cells were harvested onto glass-fiber filters using a Harvester 96 (Tomtec, Hamden, CT), and [^{3}H] thymidine incorporation (CPM) was counted on a scintillation counter (Wallac, Truku, Filand).

9. IFN-γ ELISA

To measure IFN-γ secreted by splenocytes, splenocytes were stimulated with B16F10 cells and prepared as described above. Quantities of secreted IFN-γ were measured by ELISA using an OptEIA set (Pharmingen) as described by the manufacturer. Briefly, anti-mouse IFN-γ Abs in 0.1M pH 9.5 sodium carbonate (100 ㎕/well), were plated in the wells of 96 well plates (Corning) at 100 ㎕ per well and incubated overnight at 4℃. After washing, blocking solution (10% FBS/PBS) was plated at 200 ㎕ per well and incubated for 1 hour at room temperature (RT). After washing, culture supernatants were plated at 100 ㎕ per well and incubated for 2 hours at RT.

After washing, a mixture of biotinylated anti-mouse IFN-γ Ab and streptavidin-HRP reagent were plated at 100 ㎕ per well and incubated for 1 hour at RT. After washing, substrate solution (100 ㎍/㎖ tetramethyl benzidine and 0.003% H_2O_2) was added at 100 ㎕ per well and incubated for 30 min, at RT, in the dark, followed by 4 N HCl. OD_{450} was measured on an ELISA Reader (Molecular Devices, Sunnyvale, CA, USA).

III. RESULTS

1. Analysis of DCs surface molecules. Erythrocyte-, lymphocyte-, and neutrophil-depleted bone marrow cells of C57BL/6 mice were incubated with rmGM-CSF and rmIL-4 for six days. After additional stimulation, the surface expressions of CD11c, CD40, CD80, CD86, MHC class II, and CCR7 on DCs were examined in each DCs group (**Fig. 1**). Upon flow cytometric analysis, moderate expression of CD11c was identified, which indicated that the DCs had been generated from bone marrow cells stimulated with rmGM-CSF and rmIL-4 (data not shown). DCs stimulated for 48 hours with tumor antigen plus cocktail showed moderate expression of CD80, CD86, and MHC class II. However, DCs cultured for 48 hours with no simulation, or with tumor antigen alone, showed only weak expressions of CD80, CD86, and MHC class II.

2. Analysis of Intracytoplasmic Cytokines of DCs. Intracytoplasmic cytokines (IL-12p40/p70, Il-10, and IFN-ɣ) of each DCs groups were examined by flow cytometric analysis **(Fig. 2).** However, those cytokines were not stained in any experimental group.

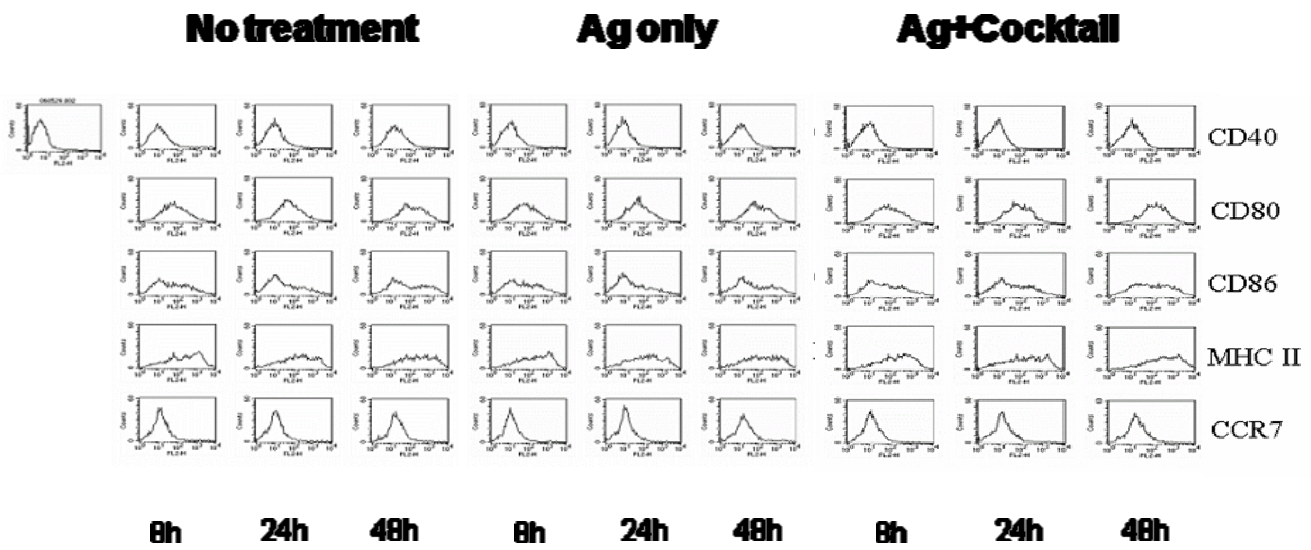

Figure 1. Analysis of DCs Surface Molecules. The surface phenotypes of each additional stimulated DCs were examined by flow cytometry using CD40, CD80, CD86, MHC class II, CCR7. The expression of surface molecules on DCs were most increased in DCs stimulated for 48 hours with tumor antigen plus cocktail.

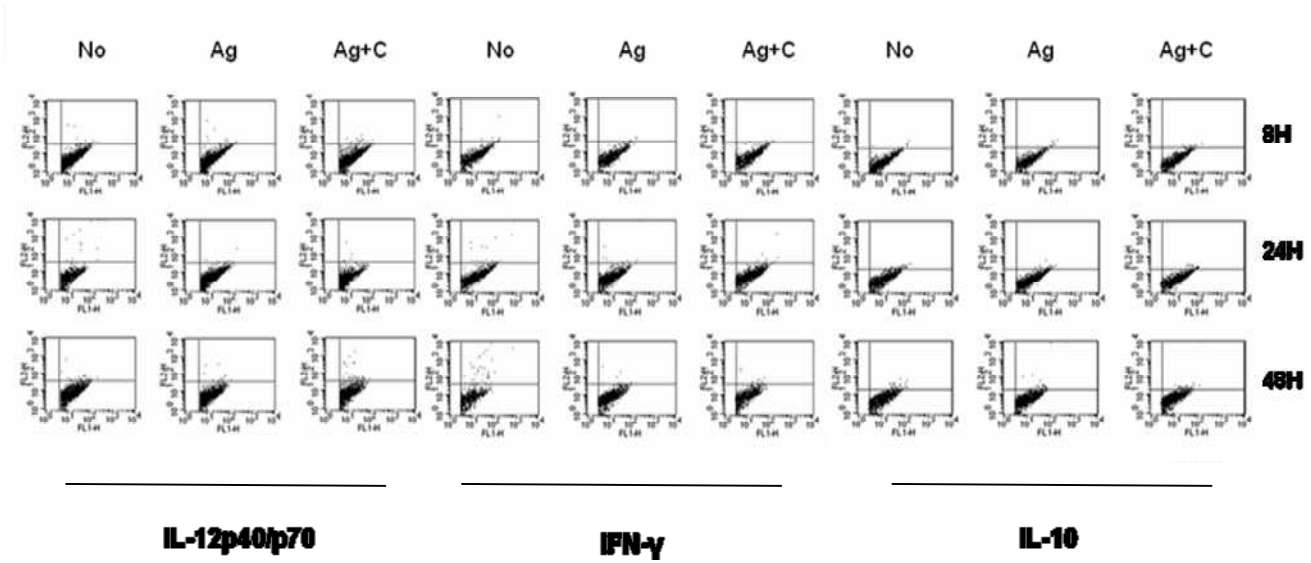

Figure 2. Analysis of Intracytoplasmic Cytokines of DCs. DCs stimulated with tumor antigen alone (group 4, 5, and 6) or tumor antigen + cocktail (group 7, 8, and 9) showed no expression of intracytoplasminc cytokines. (No;no treatment, Ag;Ag only, Ag+C;Ag+Cocktail)

3. Analysis of Cytokines Secreted by DCs. Cytokines secreted from DCs were measured by ELISA **(Fig. 3)**. IL-12p40/p70 was detected in the tumor antigen plus cocktail stimulated group. DCs stimulated for eight hours with tumor antigen plus cocktail showed marked secretion of IL-12p40/p70 (2,065 pg/㎖). As incubation time passed, the amount of secreted IL-12p40/p70 decreased (24 hours 1,120 pg/㎖ and 48 hours 598 pg/㎖, respectively). However, IL-12p40/p70 was not detected in control and the tumor antigen only stimulated groups. No groups showed IFN-ɣ, or IL-10 secretion (data not shown).

4. T cell Proliferation Assay. Isolated T cells from spleen were used for T cell proliferation assay. The strongest T cell proliferation (7,253 cpm) was observed with DCs stimulated for eight hours with tumor antigen plus cocktail. Elevated T cell proliferative reactions were also observed with DCs stimulated for 24 or 48 hours with same methods (2,943 cpm and 2,983 cpm, respectively) **(Fig. 4).**

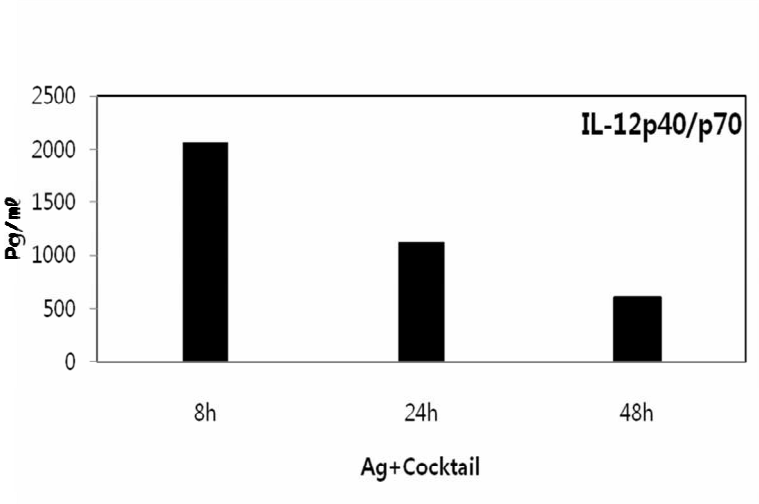

Figure 3. Analysis of Cytokines Secreted by DCs. DCS stimulated for 8 hours with tumor antigen plus cocktail showed more IL-12p40/p70 secretion (2,065 pg/㎖) than 24 hours (1,120 pg/㎖) or 48 hours (598 pg/㎖) stimulated DCs.

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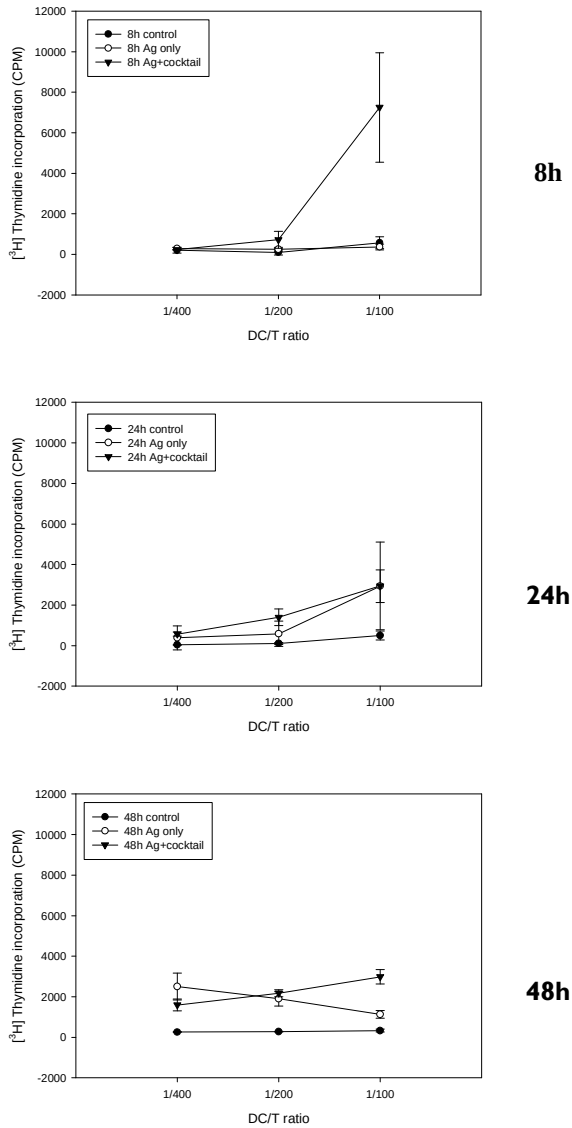


Figure 4. T cell Proliferation Assay After in vitro DCs-treatment. DCs stimulated for 8 hours with tumor antigen plus cocktail showed more T cell proliferation (7,253 cpm) than 24 hours (2,943 cpm) or 48 hours (2,983 cpm) stimulated DCs.

5. Analysis of Cytokines Secreted by DCs-stimulated T cells. IFN-γ, IL-12p40/p70, and IL-10 were examined in culture media of T cells stimulated by DCs. IFN-γ secretion was most increased with T cells stimulated by eight hours with tumor antigen plus cocktail stimulated-DCs group (186 pg/㎖) **(Fig. 5).** As stimulation time passed, the rate of secretion was reduced (139 and 132 pg/㎖ respectively). In T cells stimulated by tumor antigen only stimulated DCs, IFN-γ secretion was similar to control. IL-12 and IL-10 were hardly detectable by T cells and its secretion showed no significant differences.

6. Analysis of Tumor Sizes and Mice Survival Rate. Nineteen days after B16F10 injection to mice, tumor volume reduction was evident in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail (group Ⅳ), which showed a much reduced tumor volume(695±514 ㎣) than the control (group Ⅰ, 2,020±808 ㎣). (**Fig. 6**) Mice treated with DCs stimulated for eight hours with tumor antigen only (group Ⅱ) also showed tumor volume reduction.

The mice treated with DCs stimulated for eight hours with tumor antigen plus cocktail showed best survival rate (66 days, control 32 days) (**Fig. 7**). The groups treated with DCs stimulated with tumor antigen alone also showed increased survival rates (8 hours at 46 days, and 48 hours at 42 days, respectively), but a survival benefit was not observed in mice treated with DCs stimulated for 48 hours with tumor antigen plus cocktail (33 days).

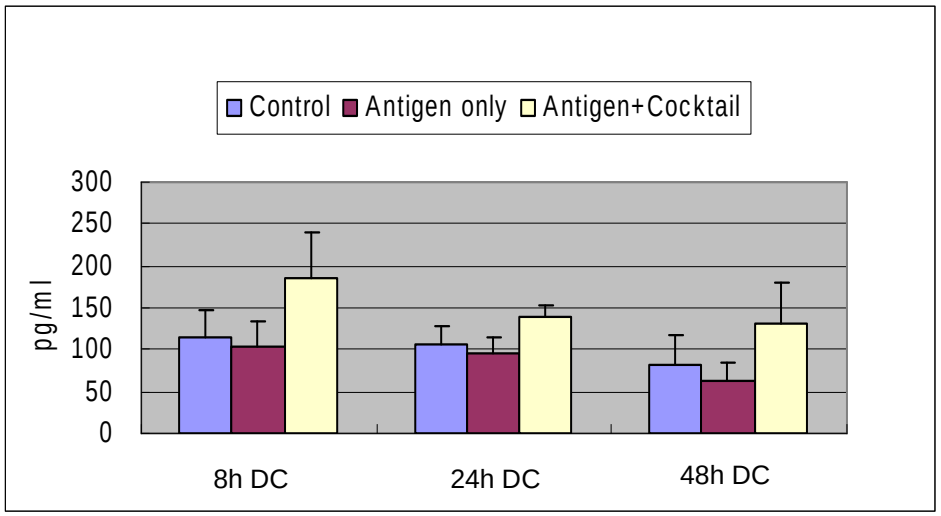


Figure 5. Analysis of Cytokines Secreted by DCs-stimulated T cells. IFN-γ secretion was most increased by T cells activated by DCs stimulated for eight hours with tumor antigen plus cocktail (186 pg/㎖).

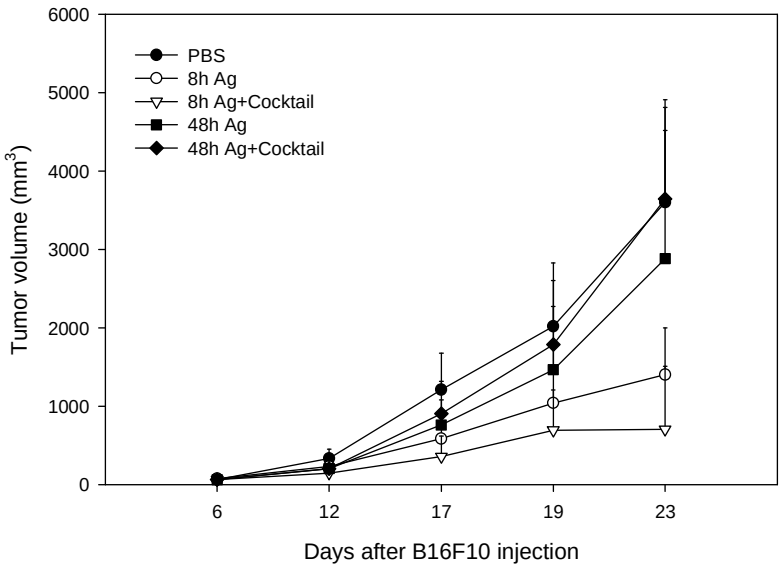


Figure 6. Analysis of Tumor Size. Tumor volume was reduced in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail (695±514 ㎣, control 2,020±808 ㎣)

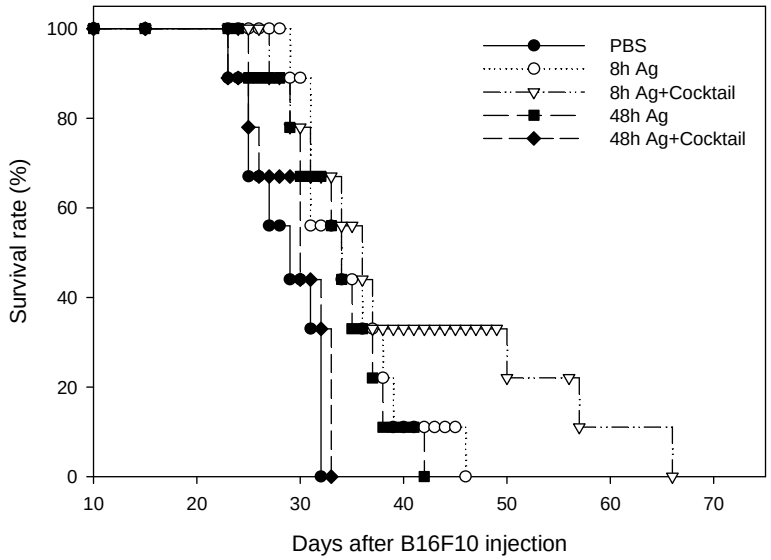
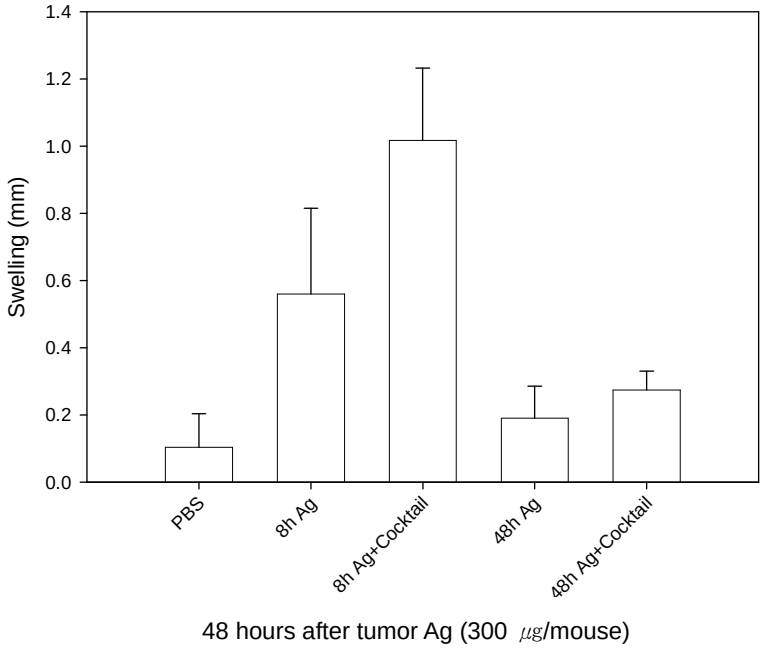


Figure 7. Analysis of Mice Survival Rate. Mice survival rate was increased in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail (66 days, control 32 days).

7. DTH. All study groups produced a DTH reaction when compared to control group. The groups treated with 8 hour-stimulated DCs showed more potent DTH than 48 hour-stimulated DCs-treated groups. The most potent DTH reaction (1.0167㎜) was observed in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail (control, 0.1033 ㎜) (**Fig. 8**)

8. T cell Proliferation Assay After DCs Immunotherapy. All study groups induced T cell proliferation *in vitro* when compared to control. Similar to the DTH reaction, 8 hour-stimulated DCs-treated groups showed stronger T cell proliferation *in vitro* than 48 hour-stimulated DCs-treated groups (**Fig. 9**). The strongest T cell proliferation (7,800 cpm) was observed in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail (control, 2,572 cpm).

9. Cytokines Secretion After DCs Immunotherapy. Similar to the DTH and T cell proliferation assay, IFN-γ secretion was increased more in groups subjected to 8 hour-stimulated DCs than 48 hour-stimulated DCs. The highest IFN-γ secretion (30,148 pg/㎖) was observed in the group treated with DCs stimulated for eight hours with tumor antigen alone (**Fig. 10).**



F***igure 8. DTH.*** Eight hour-stimulated DCs-treated groups showed stronger DTH reactions than 48 hour-stimulated DCs-treated groups.

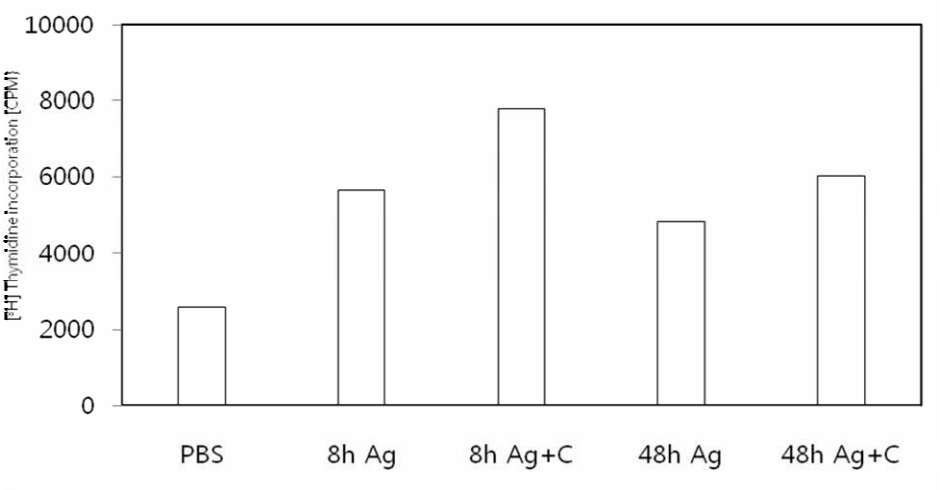


Figure 9. T cell Proliferation Assay After DCs Immunotherapy. Eight hour-stimulated DCs-treated groups showed stronger T cell proliferation than 48 hour- stimulated DCs-treated groups. (C;Cocktail)

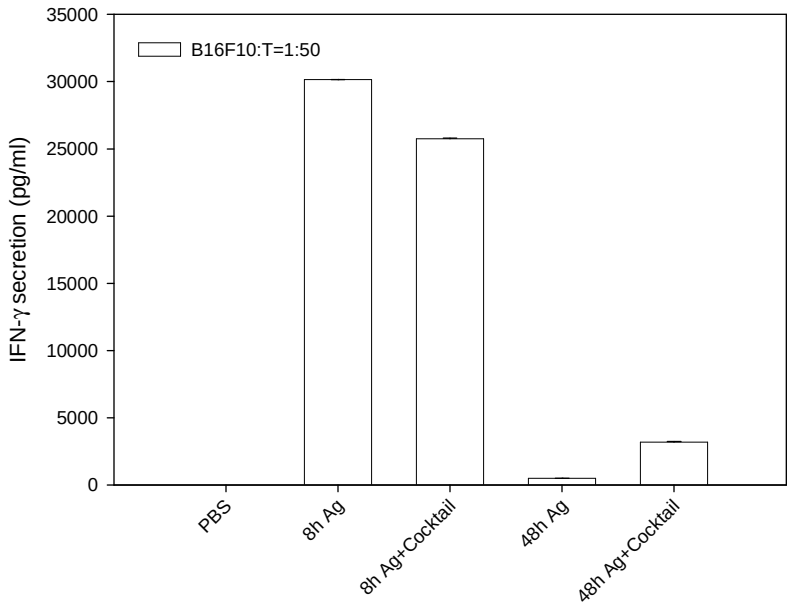


Figure 10. Cytokines Secretion After DCs Immunotherapy. IFN-γ secretion was more increased in 8 hour-stimulated DCs-treated groups than 48 hour-stimulated DCs-treated groups

IV. DISCUSSION

In this study, mouse bone marrow-derived DCs were generated and were further stimulated with tumor antigen alone or tumor antigen plus cocktail (anti-CD40 antibody + TNF-α + IL-1β) for 8 hours, 24 hours or 48 hours, respectively. Then the characteristics of DCs, such as surface molecules (CD40, CD80, CD86, MHC class II, and CCR7), cytokines (IL-12, IFN-γ, and IL-10) produced by DCs, DCs-induced T cell proliferation *in vitro*, and the production of IFN-γ by those T cells, were evaluated.

Furthermore, mice with malignant melanoma were treated with DCs stimulated with tumor antigen alone or tumor antigen plus cocktail for 8 hours or 48 hours, respectively. Then tumor size and survival rate were evaluated. By means of matching the functional characteristics of DCs with the clinical efficacy of DCs immunotherapy, we attempted to determine the optimal guideline of DCs for DCs immunotherapy in mouse melanoma.

The best clinical responses in terms of tumor volume reduction and increased survival rate were observed with DCs stimulated for eight hours tumor antigen plus cocktail. DCs stimulated for eight hours with tumor antigen alone also showed improved clinical responses, but these responses were weaker than those induced by DCs stimulated with both tumor antigen and cocktail. Thus, we examined the characteristics of DCs stimulated for

eight hours with tumor antigen plus cocktail. These DCs expressed more IL-12 than other groups. An i*n vitro* T cell proliferation assay showed the strongest response with DCs stimulated for eight hours with tumor antigen plus cocktail. IFN-ɣ secretion by DCs-stimulated T cell was greater than other groups. Immunologic monitoring such as DTH, T cell proliferation, and IFN-γ secretion by T cells in vaccinated mice were also performed to compare the immune responses to clinical responses. All immunologic monitoring method showed that 8 hour-stimulated DCs-treated groups had stronger reactions than 48 hour-stimulated DCs-treated groups. With all of these functional characteristics of DCs, clinical responses, and immunologic reaction results, we concluded that proper secretion of IL-12 by DCs is an optimal guideline of DCs for DCs immunotherapy in mouse melanoma.

In this study, the surface molecules (CD80, CD86, and MHC class II) were expressed the most in DCs stimulated for 48 hours with tumor antigen plus cocktail. However, clinical responses of this group were weaker than those induced by DCs stimulated for eight hours with same method. It is not fully known whether the status of DCs maturation is a major indicator of clinical efficacy. In some clinical studies, T cell stimulatory capacities of immature DCs are shown to be less, compared to mature DCs[15]. However, other clinical trials indicate immature DCs are superior to mature DCs, at least, with regard to the induction of T cell responses[16, 17]. Nevertheless, the consensus regarding the activation status of DCs for effective DCs

immunotherapy has not been reached. Generally, DCs stimulated with maturation factors are thought to be more potent in APC than immature DCs[18,19]. However, the APC function of DCs is thought to be limited to the later development of an immune response to avoid improper or autoimmune reaction. Several studies have shown that newly activated DCs express cell surface molecules at high levels and produce some factors required for T cell activation. However, DCs activated for long periods by maturation stimuli may not be functional immunologically [21-23].

In this study, IL-12 secretion was detected at a higher level in DCs stimulated for eight hours with tumor antigen plus cocktail. However, control DCs and tumor antigen alone stimulated DCs showed no IL-12 secretion regardless of activation time. IL-12 is known to be a cytokine that is involved directly in the generation of CTL responses[24]. One study showed that after *in vitro* activation by LPS, as well as by poly(I)-poly(C), and TNF-α plus IL-1β, DCs produce IL-12 only transiently, with a peak between 5 and 8 hours, and complete extinction after 18 hours, the time at which they become refractory to further stimulation by CD40L[23]. There are similar results in the literature using microarray techniques[25,26]. It is more intriguing that DCs activated for 8 hours preferentially induced Th1 responses, but DCs activated for 48 hours induced Th2 responses and nonpolarized T cells[23]. Eight hours-stimulated DCs were previously observed to produce IL-12 maximally and move to regional lymphoid tissue efficiently[27]. It thus appears that eight hours-

stimulated DCs induce CD4+ T cell activation[27], possibly Th1 polarization[28], and potent CTLs [29,30]. The so-called 'gold standard' mature DCs (cultured in IL-4 and GM-CSF in combination with IL-1β, IL-6, TNF-α, and PGE_2) are known to have this handicap because of their low or absent production of IL-12[31], and the concern that fully maturated DCs are unable to induce immune responses preperly[32]. These problems are supported by some studies using so-called 'gold standard' DCs, which showed relatively low levels of clinical responses[14].

To induce an Ag-specific T cell response, DCs maturation is essential[33]. Many agents have been used to induce DCs maturation experimentally such as monocyte conditioned medium[34], cytokine mixture (consisting of TNF-α, IL-6, IL-1β, and PGE_2)[35], poly(I:C) (a synthetic analog of dsRNA)[36], CpG oligonucleotides [37,38], and LPS[39]. It is known that poly(I:C), CpG oligonucleotides, and LPS show their effects on target cells by binding to endogenous TLRs (TLR3, 9, and 4)[36,40-42]. LPS-induced DCs maturation has been shown to markedly enhance the ability of Ag-loaded DCs to stimulate an Ag-specific T cell response *in vitro* and *in vivo* animal model[33,43,44]. Nevertheless, due to toxicity (potentially leading to toxic shock) and batch-to-batch variability, the use of LPS for the maturation of DCs in clinical immunotherapy is limited[45]. In this study, DCs were also stimulated with tumor antigen plus LPS for 8, 24, or 48 hours. The expression of surface marker on those DCs was higher than tumor antigen alone or tumor antigen

plus cocktail stimulated-DCs. IL-12 secretion was also increased to a greater level than DCs stimulated for eight hours with tumor antigen plus cocktail. Especially, the intracytoplasmic IL-12 was detected at a higher level in DCs treated with tumor antigen plus LPS stimulation for 8 hours whereas IL-12 was nearly undetectable in DCs treated with tumor antigen plus cocktail stimulation for 24 or 48 hours. Ultimately, however, DCs vaccines have been investigated in regard to human disease, especially human cancer. Though the use of LPS for DCs maturation is expected to enhance Ag-specific immunity[33], other substitutable clinically safe stimulation factors need to be developed.

In summary, mouse bone marrow-derived DCs were generated using different stimulation method and incubation time. Then DCs immunotherapy on a malignant melanoma mouse model was performed. After DCs immunotherapy, functional characteristics of DCs that showed the best clinical responses including reduction of tumor size and increased survival rates were evaluated. DCs stimulated for eight hours with tumor antigen plus cocktail showed the best clinical responses. Moreover, those DCs had elevated IL-12 secretion whereas others showed no IL-12 secretion. Thus, proper production of IL-12 is shown to be the optimal guideline of DCs for DCs immunotherapy in mouse melanoma.

V. CONCLUSION

In this study, functional characteristics of DCs stimulated by several maturation factors *in vitro* were examined. Then immunotherapy for murine malignant melanoma with prepared DCs was performed.

1. **The expression of surface molecules on DCs were most increased in DCs stimulated for 48 hours with tumor antigen plus cocktail.**
2. **No intracytoplasmic cytokines (IL-12p40/p70, IL-10, IFN-ɣ) were detected in all groups by flow cytometric analysis.**
3. **Il-12p40/p70 was detected in the culture supernatants of DCs stimulated for eight hours with tumor antigen plus cocktail by ELISA. However, IL-10, and IFN-ɣ were not detected in any groups.**
4. **T cell proliferation assay *in vitro* was increased by DCs stimulated for eight hours with tumor antigen plus cocktail.**
5. **IFN-ɣ secretion by DCs-stimulated T cells was induced to some degree by DCs stimulated for eight hours with tumor antigen plus cocktail.**
6. **Tumor size was reduced in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail.**

7. **Most increased survival rate were observed in the group treated with DCs stimulated for eight hours with tumor antigen plus cocktail.**
8. **Immunologic monitoring (DTH, T cell proliferation, IFN-γ secretion by T cells) showed that 8 hour-stimulated DCs are more immunogenic than 48 hour-stimulated DCs.**

Taken together, most effective immunotherapeutic results were induced by DCs stimulated for eight hours with tumor antigen plus cocktail. Consequently, proper production of IL-12 was thought to be the optimal guideline of DCs for DCs immunotherapy in mouse melanoma.

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Abstract (in Korean)

마우스 악성흑색종의 효과적인 수지상세포치료를 위한 최적조건 확립

<지도교수 이민걸>

연세대학교 대학원 의학과

조 한 경

마우스 골수세포로부터 얻은 수지상세포를 종양항원, 종양항원+칵테일(항-CD40 항체+TNF-α+IL-1β)로 각각 8 시간, 24 시간, 48 시간 동안 자극하여 수지상세포의 표면항원 (CD40, CD80, CD86, MHC class II, CCR7) 발현 정도, 수지상세포의 세포질 내 및 세포 배양액의 사이토카인 (IL-12, IFN-γ, IL-10) 분비, 수지상세포의 자극에 의한 T 세포의 증식반응 및 IFN-γ 생성 정도를 측정하였다. 또한 악성흑색종이 유발된 마우스를 각 실험군 (8 시간과 48 시간 군)의 수지상세포로 치료하여 임상적 유효성을 확인하였다. 이 후 *in vitro* 에서 측정한 수지상세포의 특성과 *in vivo* 면역치료 결과를 비교하여, 악성흑색종의 효과적인

수지상세포치료를 위한 수지상세포의 최적기준을 관찰하고자 하였다.

1) 악성흑색종에 대한 면역치료 결과 8 시간 동안 종양항원과 칵테일로 자극한 수지상세포를 주사한 실험군에서 종양크기의 감소, 생존율 증가 등의 임상적 치료결과가 가장 좋았다.

2) 8 시간 동안 종양항원과 칵테일로 자극한 수지상세포의 특성을 살펴본 결과 다른 실험군의 수지상세포에 비하여 IL-12 를 많이 생성하는 것을 관찰하였다.

이상의 결과로, 수지상세포의 충분한 IL-12 생성이 악성흑색종의 효과적인 수지상세포치료를 위한 수지상세포의 최적기준이라고 생각한다.

핵심되는말 : 수지상세포, 흑색종, 면역치료, 최적기준